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EGFR Internalization and EGFR Recycling Assays

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Updated date: May 17, 2023

An abbreviated version of this protocol was published in Frontiers in Cell and Developmental Biology in Apr 2021

ZNRF1 Mediates Epidermal Growth Factor Receptor Ubiquitination to Control Receptor Lysosomal Trafficking and Degradation

DOI: 10.3389/fcell.2021.642625

Detailed protocol

This protocol enables the detection of EGFR internalization and recycling by flow cytometry

Reagents

- EGFR-expressing cells: A549
- RPMI-1640 (Gibco, Carlsbad, CA, United States)
- Fetal bovine serum (FBS) (Gibco)
- Phosphate buffer saline (PBS)
- pH 2.8 acetate buffer (0.2 M acetic and 0.5 M NaCl)
- Sodium azide
- 4% paraformaldehyde (PFA)
- EGF (Peprotech, Rocky Hill, NJ, United States)
- Alex Flour 488-conjugated EGF (Thermo Fisher Scientific)
- Cycloheximide (CHX)
- EDTA solution

Experimental protocol

For the EGFR internalization assay:

1. A549 cells are grown in 6-cm dish (8x10^5 cells in 2 mL of RPMI containing 10% FBS).
2. Cells are washed twice with serum-free RPMI.
3. Add 2 mL serum-free RPMI for overnight starvation.
4. Cells are washed with PBS.
5. Add serum-free RPMI containing 2 μg/ml AF488-conjugated EGF and put the dish on ice for 30 minutes to stop EGFR internalization.
6. Incubate cells at 37°C for 0, 5, and 10 minutes.
7. Cells are washed with ice-cold PBS for three times.
8. Cells are washed with pH 2.8 acetate buffer at 4°C for 5 minutes.
   (Surface-bound AF488-conjugated EGF is stripped by acid)
9. Cells are washed with ice-cold PBS.
10. Detach cells from culture dish by EDTA.
11. Centrifuge for 5 minutes at 1200 RPM.
12. Cells are washed with ice-cold PBS.
13. Centrifuge for 5 minutes at 1200 RPM.
14. Cells are fixed with PBS containing 4% PFA for 20 minutes.
15. Centrifuge for 5 minutes at 1200 RPM.
16. Cells are suspended with PBS containing 2% FBS and 0.01% sodium azide.
17. Fixed cells are analyzed by flow cytometer.

For the EGFR recycling assay:

(A) To obtain the total amount of initial internalized EGFR.

1. A549 cells are grown in 6-cm dish (8x10^5 cells in 2 mL of RPMI containing 10% FBS).
2. Cells are washed twice with serum-free RPMI.
3. Add 2 mL serum-free RPMI for overnight starvation.
4. Cells are washed with PBS.
5. Cells are pre-treated with serum-free RPMI containing 10 μg/mL of CHX for 1 hour (CHX inhibits new synthesis of EGFR).
6. Add serum-free RPMI containing 2 μg/ml of AF488-conjugated EGF and 10 μg/ml of CHX, and incubate cells at 37°C for 15 minutes.
7. Cells are washed with ice-cold PBS for three times

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8. Cells are washed with pH 2.8 acetate buffer at 4°C for 5 minutes.
9. Cells are washed with ice-cold PBS.
10. Detach cells from culture dish by EDTA.
11. Centrifuge for 5 minutes at 1200 RPM.
12. Cells are washed with ice-cold PBS.
13. Centrifuge for 5 minutes at 1200 RPM.
14. Cells are fixed with PBS containing 4% PFA for 20 minutes.
15. Centrifuge for 5 minutes at 1200 RPM.
16. Cells are suspended with PBS containing 2% FBS and 0.01% sodium azide.
17. Fixed cells are analyzed by flow cytometer.

(B) To obtain the total amount of recycled EGFR.

1. A549 cells are grown in 6-cm dish (8x10^5 cells in 2 mL of RPMI containing 10% FBS).
2. Cells are washed twice with serum-free RPMI.
3. Add 2 mL serum-free RPMI for overnight starvation.
4. Cells are washed with PBS.
5. Cells are pre-treated with serum-free RPMI containing 10 μg/mL of CHX for 1 hour (CHX inhibits new synthesis of EGFR).
6. Add serum-free RPMI containing 100 ng/ml of non-labeled EGF and 10 μg/mL of CHX, and incubate cells at 37°C for 15 minutes.
7. Cells are washed with PBS for three times.
8. Add 2 mL of serum-free RPMI containing CHX, and incubate cells at 37°C for 2 hours (This step allows EGFR recycling).
9. Add serum-free RPMI containing 2 μg/ml of AF488-conjugated EGF and 10 μg/ml CHX, and incubate cells at 37°C for 15 minutes.
10. Cells are washed with ice-cold PBS for three times.
11. Cells are washed with pH 2.8 acetate buffer at 4°C for 5 minutes.
12. Cells are washed with ice-cold PBS.
13. Detach cells from culture dish by EDTA.
14. Centrifuge for 5 minutes at 1200 RPM.
15. Cells are washed with ice-cold PBS.
16. Centrifuge for 5 minutes at 1200 RPM.
17. Cells are fixed with PBS containing 4% PFA for 20 minutes.
18. Centrifuge for 5 minutes at 1200 RPM.
19. Cells are suspended with PBS containing 2% FBS and 0.01% sodium azide.
20. Fixed cells are analyzed by flow cytometer.

The ratio of recycled EGFR is determined by the total amount of recycled EGFR relative to the total amount of initial internalized EGFR.

How to cite:(Readers should cite both the Bio-protocol preprint and the original research article where this protocol was used)

1. Shen, C. and Hsu, L. (2023). EGFR Internalization and EGFR Recycling Assays. Bio-protocol Preprint. bio-protocol.org/prep2304.
2. Shen, C., Chou, C., Lai, T., Hsu, J., Lin, Y., Liu, H., Chen, Y., Ho, I., Hsu, P., Chuang, T., Lee, C. and Hsu, L.(2021). ZNRF1 Mediates Epidermal Growth Factor Receptor Ubiquitination to Control Receptor Lysosomal Trafficking and Degradation. Frontiers in Cell and Developmental Biology 0(0). DOI: 10.3389/fcell.2021.642625